Specific Nucleotide Sequence of HLA-C is Strongly Associated with Psoriasis Vulgaris

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The association of specific HLA-C nucleotide sequences with psoriasis vulgaris was investigated in 75 Japanese patients by the polymerase chain reaction method, followed by slot-blot hybridization using two specific oligonucleotide probes. The synthesized nucleotide primers were C180P, 5′-GACCGGGAGACACAGAAGTACAAG-3′ (coding for amino acid residues 61 to 68 of the α1 domain of the HLA-C molecule) and C243PR, 5′-GCTCTGGTTGTAGTAGCCGCG-3′ (residues 82 to 88), respectively. The amplified sequence detected with the probe C208A (5′-AGGCACAGGCTGACCGA-3′), including the coding region for alanine at position 73, was significantly increased in frequency in the patients compared with the healthy individuals (81% versus 48%, relative risk = 4.7, $\chi^2 = 15.3$, $p < 0.0001$). This specific nucleotide sequence is common to Cw6 and Cw7, but some other HLA-C alleles including Cw4 and C blank (Cx52) also proved to have this sequence. It is suggested that alanine at position 73 of HLA-C molecules can be a good marker for psoriasis vulgaris and that this residue may play an important role in determining susceptibility to this disease. *J Invest Dermatol 97:254–258, 1991*

It has been well recognized by standard serological typing that the association of psoriasis vulgaris (PsV) with the human leukocyte antigens (HLA) A1, B13, B17, B37, Cw6, Cw7 and DR7 is significant [1]. HLA-Cw6 has a particularly strong association irrespective of different racial or ethnic groups [1,2]. Recently, we found that HLA-A1, A2, B39, Bw46, Cw6, Cw7, and Cw11 are increased in frequency among Japanese patients with PsV; of these, especially strong associations were observed with HLA-Cw6, Cw7, and Cw11 [3]. The remaining associations could be well explained by linkage disequilibrium with Cw7 and Cw11. In addition, recent analyses of restriction fragment length polymorphism [2] and pulsed field gel electrophoresis [4] support the hypothesis of the tight linkage between HLA-C locus and the gene controlling susceptibility to PsV.

By means of direct cloning and sequencing analysis of HLA gene segments, it has been shown that certain polymorphic amino acid residues of HLA class II molecules are strongly correlated with some diseases [5]. That is, these certain amino acid residues may directly contribute to disease susceptibility by influencing the molecular structure and function of HLA class II molecules. To the best of our knowledge, however, attempts at in vitro amplification of HLA class I genes are very few [6], and DNA analysis to investigate disease associations has not been performed yet.

To determine whether specific amino acid residues of HLA-C molecules are strongly associated with PsV, we examined several known nucleotide sequences for HLA-C alleles that have been published (Fig 1). Although there were no specific sequences common to the three PsV-associated HLA-C alleles, namely Cw6, Cw7 and Cw11, it was proved that Cw6 and Cw7 shared some nucleotide sequences distinguishable from other HLA-C genes. Special attention was paid to codon 73, which codes for ^{73}Ala (alanine) instead of ^{73}Thr (threonine), because this amino acid residue is situated on the α1 domain helix that forms one side of the putative antigen-binding cleft [7]. It should be emphasized that the nucleotide sequences for some known C alleles are not yet clarified and approximately 20 to 50% individuals are untypable by specific antisera for at least one allele of HLA-C locus [8], and those unidentified antigens are designated as HLA-C blanks. In the current study, in vitro amplified specific segments of the HLA-C genes including the region encoding the 73rd amino acid residue were hybridized with specific synthetic oligonucleotide probes to determine whether this ^{73}Ala residue on HLA-C molecules is actually associated with disease susceptibility to PsV.

MATERIALS AND METHODS

Subjects Seventy-five unrelated Japanese patients (46 men and 29 women) with PsV and 50 control individuals were randomly se-

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Abbreviations:
Ala: alanine
Arg: arginine
Asp: aspartic acid
bp: base pairs
dNTP: deoxynucleoside 5′-triphosphate
EDTA: ethylenediamine tetraacetic acid
Gln: glutamine
HLA: human leukocyte antigen
PCR: polymerase chain reaction
PsV: psoriasis vulgaris
SDS: sodium dodecyl sulfate
SSPE: saline sodium phosphate EDTA
Thr: threonine
Val: valine

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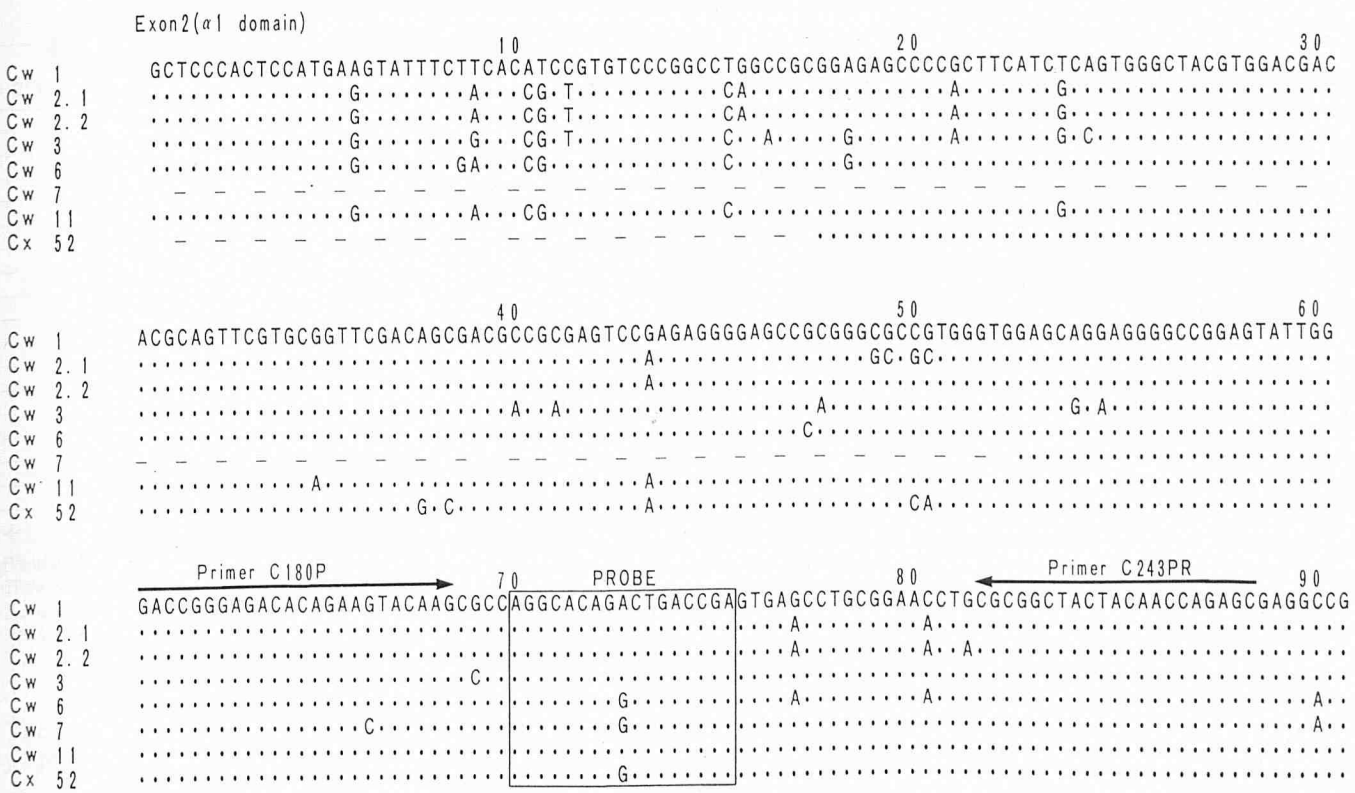

Figure 1. Published nucleotide sequences of HLA-C α1 domain (exon 2); Cw1, Cw2.1, Cw2.2, Cw3 and Cw11 [16], Cw6 and Cw7 [17,18], Cx52 [12]. *Dot*, nucleotide identical to the Cw1 sequence; *dashes*, unknown sequences.

lected for the study. All of them had their HLA class I typed serologically. HLA antigen frequencies of both the patient group and the control group are shown in Table I, which corresponded well to our previous study [3,4]. That is, Cw6, Cw7 and Cw11 were significantly increased among the patient group. (Cw6: relative risk = 14.4, $p < 0.03$; Cw7: relative risk = 2.5, $p < 0.03$; Cw11: relative risk = 3.4, $p < 0.03$.)

Methods

Oligonucleotide Primers and Probes: A set of primers were designed to anneal with HLA-C gene-specific sequences (Fig 1). The synthesized oligonucleotide primers were C180P, 5′-GACCGGGAGACACAGAAGTACAAG-3′ (coding for amino acid residues 61 to 68 of the α1 domain of HLA-C molecules) and C243PR, 5′-GCTCTGGTTGTAGTAGCCGCG-3′ (residues 82 to 88), respectively.

Two oligonucleotides were synthesized as the probes detecting a single nucleotide difference at the position encoding the 73rd amino acid; C208A, 5′-AGGCACAGGCTGACCGA-3′ (coding for ^{70}Gln ^{71}Ala ^{72}Gln ^{73}Ala ^{74}Asp ^{75}Arg) and C208B, 5′-AGGCACAGACTGACCGA-3′ (coding for ^{70}Gln ^{71}Ala ^{72}Gln ^{73}Thr ^{74}Asp ^{75}Arg). Both probes were 3′-end labelled with digoxigenin-conjugated deoxyuridine triphosphate (Dig-11-UTP; Boehringer Mannheim).

Table I. Frequency of HLA Antigens

Antigen	PsV (n = 75)	Control (n = 50)
HLA-A2	44 (59%)	25 (50%)
Bw46	17 (23%)	4 (8%)
Bw52	24 (32%)	9 (18%)
Cw6	9 (12%)	0 (0%)
Cw7	29 (39%)	10 (20%)
Cw11	17 (23%)	4 (8%)

DNA Isolation and Amplification: Genomic DNA was prepared from fresh peripheral lymphocytes by sodium dodecyl sulfate (SDS)-proteinase K-phenol/chloroform treatment in each individual and in vitro specific amplification of the HLA-C gene fragment was performed by the polymerase chain reaction (PCR) according to the method of Saiki et al [9] with minor modifications. One microgram of genomic DNA was dispensed in 100 μl of PCR mixture [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxynucleoside 5′-triphosphate (dNTP), and 50 pMole of each primer]. Two units of Taq DNA polymerase (AmpliTaq, Cetus) and 40 μl of liquid paraffin were added to each sample. They underwent 30 cycles in a programmable thermal cycler of heating to 95°C for 1 min, cooling to 55°C for 2 min, and incubation at 72°C for 1.5 min. Five microliters of the amplified reaction mixture of each sample was fractionated by polyacrylamide gel electrophoresis, and the amplified DNA fragment was visualized by ultraviolet fluorescence after staining with ethidium bromide.

Hybridization with Oligonucleotide Probes: The processed samples were denatured with 0.9 ml of 0.5 M NaOH/25 mM ethylenediamine tetraacetic acid (EDTA) and 120 μl aliquots of the mixture were applied to nylon filters (Bio Trace RP, Gelman Sciences). Filters were pre-hybridized 30 min at 45°C in 6 × saline sodium phosphate EDTA (SSPE) (1 × SSPE = 180 mM NaCl, 10 mM sodium biphosphate, 1 mM EDTA, pH 7.4), 5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrolidone, 0.02% Fi-

Table II. HLA-C Antigens and the Results of Hybridization in the Patient Group

Patient Number[a]	C Antigens[b]	C208A	C208B	Patient Number[a]	C Antigens	C208A	C208B
1	11, – (B51)	–	+	39	7, –	+	–
2	1, 7	+	+	40	–, – (Bw52)	+	+
3	3, 7	+	+	41	3, 6	+	+
4	–, – (Bw52)	+	+	42	7, 11	+	+
5	11, –	–	+	43	3, 4	+	+
6	3, 6	+	+	44	1, – (Bw52)	+	+
7	3, 6	+	+	45	11, –	–	+
8	3, – (Bw52)	+	+	46	7, – (Bw52)	+	–
9	7, 11	+	+	47	–, – (Bw52)	+	–
10	11, –	–	+	48	1, 3	–	+
11	3, – (Bw52)	+	+	49	7, – (Bw52)	+	–
12	–, – (Bw52)	+	+	50	1, 3	–	+
13	3, 7	+	+	51	3,7	+	+
14	1, –	+	+	52	7, 11	+	+
15	1, –	+	+	53	4, 7	+	–
16	–, – (Bw52)	+	+	54	8, –	+	+
17	7, – (Bw52)	+	–	55	11, – (Bw52)	+	+
18	6, 7	+	–	56	1, 3	–	+
19	7, –	+	+	57	1, –	–	+
20	6, 7	+	–	58	4, 7	+	–
21	7, 8	+	+	59	3, 6	+	+
22	–, – (Bw52, B44)	+	+	60	–, – (Bw52, B51)	+	+
23	3, –	–	+	61	4, 11	+	+
24	7, – (Bw52)	+	–	62	3, 7	+	+
25	7, –	+	–	63	3, – (Bw52)	+	+
26	7, – (B44)	+	+	64	3, 11	–	+
27	3, 7	+	+	65	1, 7	+	+
28	4, – (B44)	+	+	66	3, – (Bw52)	+	+
29	4, – (Bw52)	+	–	67	7, – (Bw52)	+	–
30	–, – (Bw52)	+	–	68	7, – (B44)	+	+
31	11, –	–	+	69	3, 7	+	+
32	6, – (Bw52)	+	–	70	3, 6	+	+
33	11, –	–	+	71	7, –	+	–
34	3, 7	+	+	72	7, 11	+	+
35	1, 3	–	+	73	1, – (Bw52)	+	+
36	4, 11	+	+	74	3, – (Bw52)	+	+
37	11, – (Bw52)	+	+	75	3, 11	–	+
38	6, 11	+	+				

[a] Men, patients 1–46; women, 47–75.
[b] Blank (– in C antigens) indicates the possibility of homozygosity for a particular allele or the presence of an unidentified allele.

coll, 0.02% bovine serum albumin), 0.02% SDS, 0.1% N-lauroyl sarcosine. Hybridization with Dig-11-UTP–labeled C208A or C208B (1.5 pMole/ml of hybridization solution) was carried out for another 30 min at 45°C, adding the equal quantity of the other unlabeled probe as a competitor. Then the filters were washed twice in 2 × SSPE/0.1% SDS for 5 min at room temperature, and then twice again in 0.3 × SSPE/0.1% SDS for 15 min at 47°C. The filters were washed with 100 mM Tris-HCl (pH7.5)/150 mM NaCl 1 min, then incubated with 0.5% Blocking reagent (Boehringer) 30 min. The filters were incubated 30 min at room temperature in a solution of alkaline-phosphatase–conjugated antibody to digoxigenin (150 mU/ml) in 100 mM Tris-HCl (pH7.5)/150 mM NaCl. Unbound antibody-conjugate was removed by washing twice in the Tris/NaCl buffer 15 min. The colorimetric reaction was carried out in 10 ml freshly prepared 100 mM Tris-HCl (pH9.5), 100 mM NaCl, 50 mM $MgCl_2$, 45 μl nitroblue tetrazolium salt (75 mg/ml), and 35 μl 5-bromo-4-chlono-3-indolyl phosphate (50 mg/ml) per 100 cm^2 filter at room temperature. The reaction was stopped by washing the filters in 10 mM Tris-HCl (pH8.0)/1 mM EDTA.

Additional Experiment: Nine patients with PsV (patients 1, 4, 11, 29, 31, 36, 53, 66, and 71 as shown in Table II) were chosen. Total RNA were prepared from fresh peripheral blood lymphocytes, and then cDNA were synthesized using random primers (Takara Shuzo) and M-MuLV reverse transcriptase (Stratagene). All the samples were amplified with the same primers and hybridized with the same probes as mentioned above.

Statistical Analysis: The Chi-square test with Yates' correction was used to determine statistical significance. The relative risks were calculated by Woolf's method.

RESULTS

All the genomic DNA samples from 75 patients and 50 controls were successfully PCR-amplified, which was confirmed by the detection of 84-base pair (bp) single bands on polyacrylamide gel following electrophoretic analysis (data not shown). The results of slot-blot hybridization with two synthetic probes clearly discriminated positive samples from negative ones. Representative blots are shown in Fig 2.

Table II shows the results of hybridization with both probes and the HLA-C antigens identified serologically in the patient group. The overall results are summarized in Table III. The frequency of the samples that hybridized with the probe C208A, which includes the sequence encoding ^{73}Ala, was significantly increased among PsV patients compared with healthy controls (81% versus 48%, relative risk = 4.7, $\chi^2 = 15.3$, $p < 0.0001$). Conversely, a significant decrease was also observed in the patient group when C208B was used as the probe, which includes the sequence encoding ^{73}Thr (79% versus 96%, relative risk = 0.15, $\chi^2 = 5.9$, $p < 0.02$).

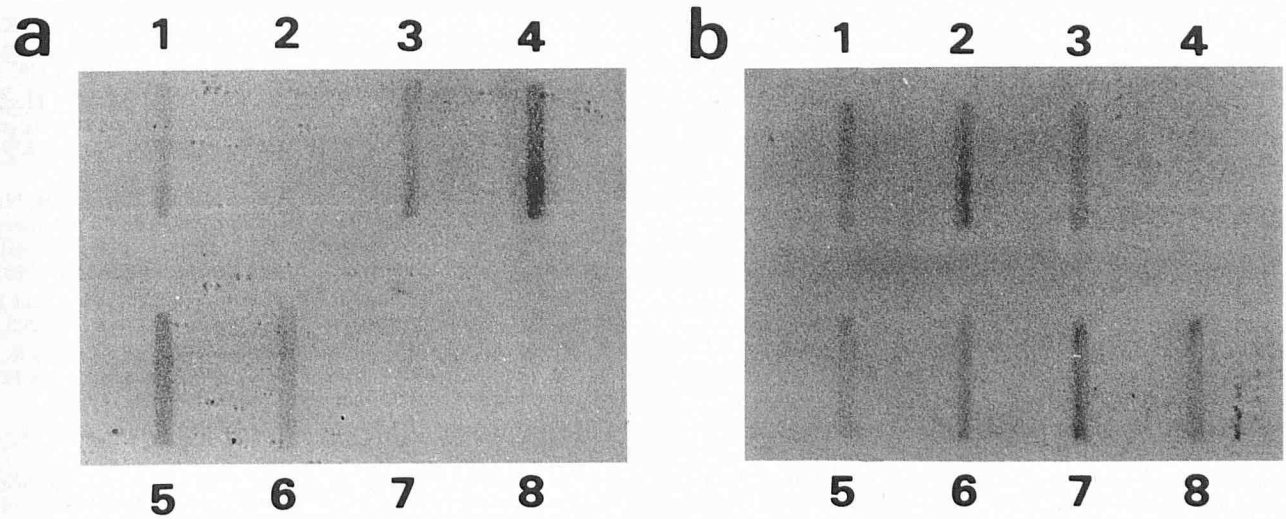

Figure 2. Representative slot-blot analysis of eight PsV patients. A specific segment of the HLA-C gene was amplified by the PCR method and hybridized with two oligonucleotide probes, C208A (*a*) and C208B (*b*). S1, Cw1/7 (patient 65); S2, Cw11/− (patient 10); S3, Cw7/11 (patient 52); S4, Cw4/− (Bw52) (patient 29); S5, Cw4/11 (patient 61); S6, Cw3/7 (patient 3); S7, Cw1/3 (patient 56); S8, Cw11/− (patient 5). C208A is positive for S1, S3, S4, S5, and S6. C208B is positive for S1, S2, S3, S5, S6, S7, and S8. (S = sample number in Fig 2; patient numbers are as in Table II.)

The hybridization patterns of PCR-amplified cDNA with the same probes were in accord with those shown in Table II.

DISCUSSION

The analysis of PCR-amplified HLA-C gene segments from 75 PsV patients and 50 healthy controls with two synthetic sequence-specific oligonucleotide probes revealed a strong association between PsV and alanine at position 73 on the α1 domain helix of HLA-C molecules. The results of the hybridization of a total of 125 individuals correlated perfectly with HLA-C antigens identified serologically and whose DNA sequences are known. Moreover, the additional experiment utilizing cDNA showed the same hybridization patterns. These facts indicated that PCR error was not likely to have occurred and that we probably successfully amplified C locus genes and not pseudogenes. It also became apparent that C blank genes were actually transcripted.

Although the unique substitution of ^{73}Ala instead of ^{73}Thr is common to Cw6 and Cw7, some other HLA-C antigens, including C-blanks, also proved to share this substitution, suggesting their possible association with PsV. Interestingly, the search for other HLA class I loci demonstrated that the C-blank antigen associated with Bw52 possessed ^{73}Ala. In the Japanese population, A24-Bw52-C blank-DR2-Dqw1 haplotype occurs relatively often [10,11], and this C-blank gene is tentatively termed Cx52, although this association is unclear because of the lack of specific alloantisera. However, a partial DNA sequencing of this gene has been performed recently [8,12], revealing the codon for this position being the same as that of Cw6 and Cw7. It is likely from our results that the linkage of C-blank, or Cx52 with this haplotype really exists, and all our cases of C-blank with Bw52 are most probably Cx52.

Furthermore, some of the C-blank antigens other than Cx52 also proved positive for ^{73}Ala. It became evident that Cw4, which can be defined serologically and whose gene has not been analyzed yet, had this residue. On the other hand, it proved that Cw8 and some C-blank antigens posessed ^{73}Thr. The linkage of C blanks with B44 or B51 is also commonly observed in the Japanese population [10,11], and in either case the linked C-blank antigen possessed ^{73}Thr.

It should be emphasized that Cw11 is also strongly associated with Japanese patients with PsV [3]. The fact that Cw11 antigen does not possess ^{73}Ala indicates that another sequence may be involved in determining susceptibility to PsV. It was found that 92% of patients were ^{73}Ala or Cw11 positive as compared with 58% in controls. ($\chi^2 = 20.4$, relative risk = 8.3, $p < 0.00001$.)

According to the three-dimensional model of HLA-class I molecule, the 73rd amino acid is situated on one side of the putative antigen-binding cleft, as described previously [7]. Although the possibility that other linked genes other than HLA-C may contribute to PsV cannot be overlooked [13], this amino acid is, at least, a good marker for this disease. To elucidate whether ^{73}Ala is really an important amino acid determining susceptibility to PsV, it is necessary to identify triggering agents and analyze T-cell clones recognizing the MHC class I-antigen complex.

Finally, we searched for amino acid sequences homologous to this putative disease determinant of HLA-C molecules in the protein identification resource protein sequence database using Pearson and Lipman's method [14]. In our computer search, we found that the sigma 2 protein of reovirus contains the sequence Gln Ala Gln Ala Asp Arg Val, which is identical to this portion (amino acid residues 70–76) of the gene product of HLA-C [15]. At present, however, the biologic significance of this similarity is unknown. More extensive studies are required to clarify the possible differences of disease patterns (clinical course, heredity, age of onset) between ^{73}Ala positive patients and Cw11 positive patients.

Table III. Frequency of the Specific Sequences

Probe	Psoriasis Vulgaris (n = 75)	Controls (n = 50)	Relative Risk	p
C208A (^{73}Ala)[a]	61 (81%)	24 (48%)	4.7	< 0.0001
C208B (^{73}Thr)	59 (79%)	48 (96%)	6.5	< 0.02

[a] Individuals positive for Cw6 and/or Cw7; psoriasis vulgaris 36 (48%): control 10 (20%). Relative risk = 3.7, $p < 0.002$.

Note Added in Proof: We noticed that by using the primers C180P and C243PR, there is a possibility of amplifying some HLA-B genes. We designed a new primer C133P, 5′-GGGAGCCGCGGGCGCCGTGGGTG-3′ (coding for residues 45 to 52) and amplified the DNA samples with the primers C133P

and C243PR. The amplification condition was the same except for 1) addition of 2.5 μl formamide and 2) 62°C as the annealing temperature. Although there was no change on the results described above, we recommend this new set of primers for further experiments.

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